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Members of the U.S. Armed Forces receive immunization with vaccinia virus vaccine. Efforts to develop a safe and effective tissue culture-derived vaccinia vaccine to replace the standard vaccine produced by scarification on cows' skin have advanced early clinical studies. It is generally accepted that protection induced by the conventional vaccinia vaccine correlated with cellular immune responses. The purpose of this research program is to measure human cellular and humoral immune responses to live experimental and standard vaccinia vaccines.

During the first year of this contract we have: (1) prepared live and killed vaccinia virus antigens and (2) established vaccinia virus-specific T cell assays; (3) obtained, separated and cryopreserved peripheral blood mononuclear cells from 92 vaccinees in a clinical study with experimental and standard vaccinia vaccines at Ft. Sam Houston, Texas, (4) performed lymphocyte proliferation assays on the day 0 and day 27 samples from these vaccinees. A majority of the vaccinees developed vaccinia virus specific T cell responses to the experimental vaccine, which was given intradermally or intramuscularly; however, almost all of the recipients developed specific T cell responses to the standard vaccine, given by scarification.

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FOREWORD

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(✓) In conducting research utilizing recombinant DNA technology,the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Francis A. Ennis
Principal Investigator's Signature

6/28/95
Date

TABLE OF CONTENTS

INTRODUCTION

There is a great deal of interest in the potential use of vaccinia virus as a vector for recombinant vaccines against human and veterinary diseases. Despite the success of vaccination in the eradication of smallpox and evidence that cellular immune mechanisms are essential for virus elimination, little is known about cellular immune responses to vaccinia virus. In addition, vaccinia virus-specific, HLA-restricted cytotoxic T lymphocytes (CTL) had not been demonstrated in man (1,2) although virus-specific CTL have been detected to many other human viruses. Studies with murine models have demonstrated the presence of vaccinia virus-specific CD8+ CTL responses that are MHC class I-restricted (3,4). Studies in different animal species demonstrate that these virus-specific CTL responses correlate with recovery from pox virus infections (1,3,5-6), but did assess the potential effector contribution of CD4+ CTL as part of the recovery process. Recently our laboratory described for the first time human vaccinia specific CD4+ and CD8+ CTL responses (7,8). Nothing is known about the epitopes on vaccinia virus that are recognized by human T cells.

In man, the significance of vaccinia-specific cell-mediated immune responses has been demonstrated by studies of vaccinated children who had thymic aplasia. Some of these children died of progressive vaccinia after accidental immunization despite producing anti-vaccinia antibodies and treatment with massive doses of vaccine immune globulin (9,10). Individuals with profound T cell defects associated with Wiscott-Aldrich syndrome also developed disseminated infection after vaccination with vaccinia virus (11). These and other reports (12,13) indicate that antibody production is not sufficient in protecting man from the complications of smallpox vaccination.

Certain members of the U.S. Armed Forces continue to receive immunization with standard vaccinia virus vaccine. Only a limited amount of stored vaccine remains available. There is a desire to produce a modern tissue-culture derived vaccine, to replace the standard vaccine produced by the old methods of scarification of the skin of cows. Efforts to develop a safe and effective modern tissue culture-derived vaccinia vaccine have advanced to the point of early clinical studies. In addition to the need for a safe and effective alternative to the current vaccinia vaccine, the new vaccinia vaccine is likely to be used as a vector carrying gene(s) encoding for protective protein antigens to immunize against other agents with vaccinia as a safe and potent carrier.

It is generally accepted that protection against smallpox induced by the conventional vaccinia vaccine correlated with restriction of lesion size to skin challenge with vaccinia virus vaccine. Therefore, it is important to be certain that newly developed vaccinia vaccines induce cellular immunity.

BODY

We have completed all of the experiments that were scheduled for year 1 as outlined in the statement of work. A description of the experiments and results are outlined below:

1. Preparation of vaccinia virus antigens

2. Preparation of live vaccinia virus pools

Plates of CV1 cells were infected with vaccinia virus (NYCBH) at a MOI of 10:1 for 2 hours and media was added and plates were incubated overnight. Plates containing control CV1 cells not infected with vaccinia virus were also established. The next day the vaccinia virus infected plates demonstrated cytopathic effects (CPE). Supernatant fluids were discarded and the cells were removed by scraping, resuspended in medium, sonicated and pelletted and aliquots of the remaining supernatant were frozen at -70°C to be used as live virus. The titer of this live vaccinia virus pool is $6.3x10^8$ PFU/ml Aliquots of supernatants from similarly treated infected and control cells were boiled for 10 min and then frozen at -70° to be used as inactivated virus and control antigens. The boiled aliquot from the virus infected supernatant was later tested for residual infective virus on CV1 cells and no CPE was detected.

3. Establishment of vaccinia virus-specific T cell assays

4. Identification of normal vaccinia virus immune donors with high T cell responses to vaccinia virus antigens.

Three healthy adults who had been reimmunized with vaccinia virus approximately three months, three years, or seven years previously, as well as a vaccinia-naive donor, supplied PBMC for us to perform preliminary experiments to gain information about the specificity and sensitivity of our vaccinia specific T cell lymphoproliferation assays. These donors were laboratory workers who were immunized because they were working with vaccinia recombinant viruses and the non-vaccinia immune donor was a laboratory employee who was not working with vaccinia virus. These individuals donated blood as part of an IRB approved protocol at the University of Massachusetts.

Briefly, proliferation assays were performed in which $2x10^5$ PBMC were added per well and replicates of 3 wells each were exposed to:

Control antigen diluted 1:20, 1:100, 1:500, 1:2500
Killed vaccinia antigen diluted 1:20, 1:100, 1:500, 1:2500
Live vaccinia virus at an MOI of 1, 0.5, 0.25, 0.125

The medium used was RPMI +10% heat inactivated human AB serum (ABI). On day 4 we added tritiated thymidine to the wells and incubated for 15 hrs longer. On day 5 the plates were harvested using a Skatron cell harvester and radioactivity was counted in an LKB beta plate reader.

The results shown below in Table 1 demonstrate that the PBMC of all three donors had relatively little lymphoproliferation following exposure to the control CV1 antigen. A very brisk lymphocyte proliferative response was detected using the killed vaccinia antigen with stimulation indices of approximately 100:1 at multiple antigen dilutions for each of the three donors. Somewhat lower but very convincing proliferative responses were also observed using PBMC exposed to the live virus antigen. Because of the availability of a relatively large number of PBMC from the donor immunized 7 years ago and the very convincing proliferative responses to both live virus and inactivated antigen (stimulation indices of over 100:1 and about 45:1 respectively) we decided to use the PBMC of this donor in subsequent experiments.

Table 1. Proliferation of human immune PBMC to Vaccinia Antigens

	PBMC of Donors who were revaccinated after					
	3 months		7 years		3 years	
Antigen	CPM	SI	CPM	SI	CPM	SI
Medium Control	196.95		172.23		592.51	
Live virus (M01)						
1.0	868.9	4.4	1631.9	9.5	23664.0	39.9
0.5	577.4	2.9	7774.5	45.1	27381.3	46.2
0.25	3467.4	17.6	8336.8	48.4	16008.6	27.0
0.125	1912.8	9.7	5535.6	32.1	16836.5	28.4
Killed virus						
1:20	23605.3	119.9	31197.2	181.1	69429.6	117.1
1:100	18814.8	95.5	23151.9	134.4	44460.1	75.0
1:500	10616.7	53.9	17552.0	101.9	30977.8	52.3
1:2500	4081.1	20.7	3628.1	21.1	19833.5	33.5
Control Ag						
1:20	335.3	1.7	812.8	4.7	1183.0	2.0
1:100	354.2	1.8	538.0	3.1	670.9	1.1
1:500	324.4	1.7	358.6	2.1	632.7	1.1
1:2500	311.0	1.6	206.7	1.2	555.3	.9

Following the demonstration of brisk lymphoproliferative responses to both live and inactivated vaccinia antigens but not to the control cell antigen we tested the PBMC of a young adult who had no history of being immunized with vaccinia vaccine. The results in Table 2 demonstrate that the PBMC of the non-vaccinated donor were not stimulated by either the live virus or the inactivated vaccinia antigen preparation. The positive control vaccinia-immune donor's PBMC were stimulated to a significant degree by both the inactivated and live vaccinia virus antigen preparations.

Table 2. Proliferative Response of PBMC for Vaccinia Non-Immune and Immune Donor

	Non-Immune		Immune	
	CPM	SI	CPM	SI
Medium Control	3057.1		354.5	
Live virus (MOI)				
.8	2577.4	0.8	2877.8	8.1
.4	3136.2	1.0	2529.1	7.1
.2	2454.1	0.8	4469.6	12.6
.1	2666.5	0.9	6008.0	16.7
Killed virus Ag				
1:40	15086.0	1.6	25535.3	31.4
1:80	12963.8	1.2	23650.1	25.1
1:160	8800.5	2.0	18552.3	31.6
1:320	10378.4	3.6	18358.7	50.0
Control Ag				
1:40	9399.2		813.3	
1:80	10793.5		940.9	
1:160	4391.0		587.8	
1:320	2917.8		367.1	

5. The acquisition, separation, and cryopreservation of peripheral blood mononuclear cell (PBMC) from paired samples from all vaccinees (92 total volunteers) on day 0 and 4 weeks post inoculation in the phase II clinical trial sponsored by USAMRIID at Fort Sam Houston.

Two of our scientists made 5 trips to Ft. Sam Houston, San Antonio Texas to obtain and process specimens of human PBMC required for testing lymphocyte responses to the experimental vaccinia vaccine being tested in volunteers by the U.S. Army scientists. The number of volunteers whose PBMC were obtained, processed and cryopreserved in liquid nitrogen was 32, 57 and 22 on three pre-vaccine groups, and 31, 47 and 24 in the three post-vaccine groups. In total the number of paired serum samples was 92, because 19 donors did not have a second blood sample obtained.

Briefly, up to 9 vacutainers of CP Tubes (Becton Dickinson Cat #362761) were filled per donor bleed. The tubes were centrifuged at 1500 g x 20 minutes at room temperature in a horizontal rotor (swing-out). The tubes were inverted twice, and caps were removed in a laminar flow hood. Cells and plasma were transferred into 2 x 50 ml centrifuge tubes (Falcon #2070) at 35 ml/tube and were transported back to UMMC in Worcester. At UMMC at Worcester, MA (the next day after blood was obtained), the tubes were centrifuged at 400 g x 5 min. Seven ml of

plasma/donor/bleed was placed in a vial and stored at -20°C. After the remaining fluid was aspirated, the cells were resuspended in 25 ml PBS pH 7.2 (Gibco Cat. #20012-019) and centrifuged at 300 g x 5 min. Cells were then resuspended in 24 ml of RPMI and were recentrifuged. The cell pellet was then resuspended in 5 ml RPMI, 20% heat inactivated FBS and 10% DMSO. One ml was added/cryovial (Corning #25704), 5 vials/donor/bleed, and were frozen in liquid nitrogen with a rate controlled freezer (Cryomed Model 801).

6. Bulk culture lymphocyte proliferation assays on day 0 and 27 samples from all vaccinees in this trial.

We have completed under code the vaccinia-specific T lymphocyte proliferation responses on all of the volunteers' day 0 and day 27 PBMC samples. A copy of those results was sent to Drs. A. Schmaljohn and McClain who subsequently sent a copy of the vaccine group code to us. We have tabulated the responses according to each vaccine study group and developed an overall summary. Copies of these results are contained in Tables 4-8 and the raw data was sent on computer disc to Dr. A. Schmaljohn at USAMRIID. Individuals in all of the vaccine groups had vaccinia-specific T lymphocyte responses, and the three groups that received the TSI-GSD241 vaccine had similar responses whether the vaccine was given intradermally, with or without an alcohol wipe, or intramuscularly. The standard vaccine given by scarification induced T lymphocyte responses in virtually all of the vaccinees. We measured responses to both infectious vaccinia virus and to inactivated vaccinia virus antigens. The results are summarized in Table 3 and all the data are shown on the following pages.

Table 3. Vaccinia-Specific Memory T lymphocyte Proliferation Responses in Ft. Sam Houston Study, 1994

Group	Vaccine	Route	Alcohol wipe	#	Percent of Increases* in Vacc.-Spec. T Cell Responses to Live Virus	Killed Virus
1A	TSI-GSD 241	ID	+	17	41	71
1B	TSI-GSD 241	ID	-	16	50	69
2	TSI-GSD 241	IM		26	54	77
3	Wyeth	SCAR		32	91	100

* A positive proliferation response is defined as a S.I. at day 27 that is ≥ 2 fold above day 0 and also is SS at level of $p<0.05$. Three volunteers who had a ≥ 2 fold increase in CPM but p was not <0.05 to live virus but had ($p<0.001$) to killed virus are considered to be positive responders to virus. The three different vaccine groups contained one each of these type of responders.

7. In addition to completing the items 1-6 in the Statement of Work, we have completed and submitted a manuscript for publication entitled: " Human cytotoxic T cell memory: Long lived responses to vaccinia virus" by Walter Demkowicz, Rebecca Littaua, Jianming Wang, and Francis A. Ennis. A copy of the manuscript is included in the Appendix.

TABLE 4 GROUP 1A=TSI-GSD 241 INTRADERMALLLY (5.0 LOGS) WITH ALCOHOL WIPE T CELL PROLIFERATION						PRNT**** 50% PROBIT	
UMMC#	ARMY#	VIRUS	> 2 FOLD	AG	> 2 FOLD	PRE	D27
19	1	5.4	Y **	4.7	Y ***	3	3
12	17	1.5		8.2	Y **	15	5
*	20	1.3		1.5		11	44
21	29	1.2		3.3	Y **	30	41
31	31	1		3.6	Y **	3	5
30	43	1		2.3	Y **	3	N/A
66	253	2.6	Y ***	3.9	Y ***	17	76
52	261	1.5		1.4		3	3
72	267	1		1.7		5	5
58	273	4.4	Y *	15.2	Y ***	5	3
83	280	1.9		5.8	Y ***	3	198
35	289	2.7	Y *	1		3	5
34	300	2.3	Y **	4.7	Y ***	5	25
78	305	1		2.1	Y **	11	16
92	313	2.8	Y ***	9.6	Y **	3	35
100	322	2.3	Y *	2.7	Y ***	3	53
111	333	0.8		1		3	3

*=p<.05
**=p<.01
***=p<.001

**** PLAQUE REDUCTION NEUTRALIZATION TITERS OBTAINED FROM DR. D. MCLAIN, USAMRIID

TABLE 5 GROUP 1B=TSI-GSD 241 INTRADERMALLY (5.0 LOGS) WITHOUT ALCOHOL WIPE T CELL PROLIFERATION						PRNT**** 50% PROBIT	
UMMC#	ARMY#	VIRUS	> 2 FOLD	AG	> 2 FOLD	PRE	D27
27	4	1.8		1.4		3	51
4	10	2	Y ***	10.5	Y ***	3	11
8	15	4.8	Y ***	77.3	Y ***	5	57
29	22	3.7	Y	17.8	Y ***	5	95
17	27	4.3	Y **	0.7		5	18
5	35	1.4		5.1	Y *	3	3
80	263	1		2.6	Y *	13	3
63	271	12.7	Y ***	6	Y ***	3	640
79	281	1.1		1.5		3	5
87	283	1		3.1	Y *	3	12
44	292	27.6	Y ***	48.3	Y ***	5	277
40	304	18.4	Y ***	64	Y ***	3	167
48	309	8	Y ***	10.8	Y ***	3	347
96	318	1.5		1.9		3	3
97	319	1		9.1	Y ***	3	229
108	330	1.6		1.6		3	5

*=p<.05
**=p<.01
***=p<.001

**** PLAQUE REDUCTION NEUTRALIZATION TITERS OBTAINED FROM DR. D. MCLAIN, USAMRIID

TABLE 6

GROUP 2=TSI-GSD 241 INTRAMUSCULARLY (5.0 LOGS) T CELL PROLIFERATION						PRNT**** 50% PROBIT	
UMMC#	ARMY#	VIRUS	> 2 FOLD	AG	> 2 FOLD	PRE	D27
13	5	5	Y	16.3	Y ***	3	3
3	9	1		1.9		3	3
26	16	2.5	Y *	1.4		31	46
7	18	8.2	Y **	3.6	Y ***	109	100
18	24	1.9		2.1	Y **	3	3
28	28	5.7	Y **	11.2	Y ***	25	N/A
15	30	1		3.4	Y ***	3	3
6	33	1.5		45.3	Y **	6	44
23	37	9.5	Y ***	5.1	Y ***	94	50
46	251	23.5	Y ***	25	Y ***	3	128
39	257	1.8		9.5	Y ***	16	9
69	260	11.1	Y ***	15.6	Y ***	18	69
81	264	1.1		6.9	Y ***	3	3
70	265	1		5.3	Y	3	37
65	270	5.1	Y ***	1.5		3	5
55	272	5.2	Y **	5.6	Y ***	5	28
75	277	2.1	Y **	6.3	Y ***	3	32
60	282	13.9	Y ***	29.1	Y ***	12	3
54	287	4.8	Y ***	7.8	Y ***	3	42
53	288	7.4	Y ***	5.2	Y ***	3	65
82	290	1.3		8	Y **	5	3
42	293	5	Y ***	10.5	Y ***	3	5
45	295	2.5	Y ***	2	Y **	14	5
68	298	2.7	Y	1.7		5	20
50	301	0		0		16	17
77	310	1.4		8.3	Y ***	5	26

*=p<.05
**=p<.01
***=p<.001

**** PLAQUE REDUCTION NEUTRALIZATION TITERS OBTAINED FROM DR. D. MCLAIN, USAMRIID

TABLE 7

GROUP 3=WYETH DRYVAX BY SCARIFICATION
T CELL PROLIFERATION

UMMC#	ARMY#	VIRUS	> 2 FOLD	AG	> 2 FOLD	PRNT**** 50% PROBIT PRE	PRNT**** 50% PROBIT D27
24	3	5.5	Y ***	5.4	Y ***	68	256
11	12	7	Y ***	7.6	Y ***	5	362
22	13	3.1	Y **	5.4	Y ***	3	137
16	21	25	Y *	23.9	Y ***	3	63
10	23	1		2.4	Y *	3	3
32	25	3.1	Y *	19.8	Y *	20	1516
2	32	7.8	Y ***	7.9	Y ***	5	727
9	34	19.8	Y **	45.4	Y ***	3	299
20	40	2.4	Y	64.1	Y ***	3	97
25	42	16.8	Y ***	44.7	Y ***	3	114
64	254	6.1	Y ***	3.4	Y ***	8	68
85	258	27.7	Y ***	38.9	Y ***	3	286
84	259	11.3	Y ***	35.8	Y ***	5	712
61	262	12.8	Y ***	21.7	Y ***	3	416
86	266	12.9	Y ***	18.8	Y ***	5	227
49	268	5.4	Y ***	8.9	Y ***	3	162
57	274	3.6	Y ***	16.8	Y ***	5	104
59	275	6.6	Y ***	7.8	Y ***	3	70
76	278	1.7		2.9	Y ***	19	70
62	279	5.3	Y ***	3.6	Y ***	5	135
73	294	23.9	Y ***	36.2	Y ***	5	664
37	297	47.8	Y ***	34.5	Y ***	5	615
43	299	8.6	Y ***	8.7	Y ***	119	315
41	302	17.5	Y ***	4	Y ***	3	346
38	303	16.1	Y ***	23.9	Y ***	7	206
88	307	3.4	Y *	36.2	Y ***	3	687
90	311	6.8	Y ***	13.9	Y ***	3	164
93	314	4	Y ***	4.9	Y ***	3	46
98	320	2.9	Y ***	3.3	Y ***	3	74
102	324	2.6	Y **	6.3	Y ***	3	119
104	326	12.1	Y ***	13.3	Y ***	3	48
106	328	1.9		4.5	Y ***	3	44

*=p<.05
**=p<.01
***=p<.001

**** PLAQUE REDUCTION NEUTRALIZATION TITERS OBTAINED FROM DR. D. MCLAIN, USAMRIID

TABLE 8

GROUP SCR=TSI-GSD 241 BY SCARIFICATION
T CELL PROLIFERATION

UMMC#	ARMY#	VIRUS	> 2 FOLD	AG	> 2 FOLD	PRNT**** 50% PROBIT PRE	PRNT**** 50% PROBIT D27
94	315	1		2.4	Y**	3	3
95	316	1		1.5		5	167

**** PLAQUE REDUCTION NEUTRALIZATION TITERS OBTAINED FROM DR. D. MCLAIN, USAMRIID

CONCLUSIONS

The bulk culture lymphocyte proliferation data obtained during the first year of this study indicate that the experimental in vitro tissue-culture derived vaccine that scientists at USAMRIID developed is immunogenic in most recipients; however it is less immunogenic than the standard smallpox vaccine. It is difficult to make a direct comparison between the two vaccines because the TSI-GSD 241 was administered by the intradermal or intramuscular routes and the standard vaccine was administered by scarification. The results (summarized in table 3) indicate that individuals in all of the vaccine groups had vaccinia-specific T-lymphocyte responses. The three vaccine groups that received the TSI-GSD241 vaccine had similar vaccinia specific immune responses, whether the vaccine was given intradermally, with or without an alcohol wipe, or intramuscularly. The standard Wyeth vaccine given by scarification induced more vigorous T-lymphocyte response in virtually all of the volunteers in that group.

The plaque reduction neutralizing antibody titer values shown on the Tables with the lymphocyte proliferation data were provided to us by Dr. D. McClain of USAMRIID. The plaque neutralizing antibody responses appear to correlate with the vaccinia virus specific T cell memory responses we detected. Generally, the standard Wyeth vaccine stimulated a more vigorous humoral immune responses, as well as T lymphocyte responses, than the TSI-GSD241 vaccine.

A direct comparison of the same routes of administration between the standard Wyeth vaccine and the TSI-GSD241 vaccine was not done, i.e. the desire to develop a vaccine that would be given by injection so as to avoid safety and transmission concerns which have occurred after admnistering standard vaccine by the recommended scarification route. It is possible that the more modest immune responses induced by the TSI-GSD241 vaccine may be due to the routes of immunization and it may have stimulated a more vigorous immune response had it been administered by scarification. Alternatively, the propagation of the experimental vaccine in tissue culture cells may have caused it to become more attenuated than the in vivo propagated standard Wyeth vaccine. In summary, during the first year of this contract we have determined that the experimental TSI-GSD241 vaccine stimulates cellular immune responses when administered by the intradermal or intramuscular routes, however, the immune responses stimulated by this vaccine were lower than those generated by scarification with the standard Wyeth vaccine.

During the next year of this study we will: (1) examine the magnitude of the vaccinia virus- specific CD4+ and CD8+ cytotoxic T lymphocyte (CTL) responses in the PBMC from selected vaccinees; (2) measure the vaccinia-specific antibodies by Western blot in all the vaccinee paired serum samples; (3) measure IFN, sCD4, sCD8, and sIL-2r from vaccinia virus stimulated PBMC from selected vaccinee samples; and (4) using PBMC of selected high responders, attempt to identify several proteins of vaccinia virus that stimulate human vaccinia specific T lymphocyte responses in vitro.

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HUMAN CYTOTOXIC T CELL MEMORY:

LONG LIVED RESPONSES TO VACCINIA VIRUS

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SUMMARY

Peripheral T Lymphocytes can be classified into two groups; naive and memory T cells. The focus of this study was to examine the duration of T cell memory in humans. Vaccinia virus replicates in the cytoplasm of infected cells and is not thought to persist or become latent after acute infection. We identified long-lived vaccinia virus-specific *memory* cytotoxic T cells in adults who had been immunized against smallpox as children. Initially we detected vaccinia virus-specific T cells in PBMC while screening for HIV-1 specific T cell responses in HIV-1 seropositive subjects. These individuals had not had contact with vaccinia virus since their primary immunization in early childhood. Several vaccinia virus-specific CD4+ T cell clones were derived and characterized from these donors. Healthy HIV-1 seronegative donors who had been immunized against smallpox many years earlier (35-50 years) were also screened for vaccinia virus-specific T cell immunity. We demonstrate significant CD8+ and CD4+ cytotoxic T cell responses to vaccinia virus after in vitro stimulation, indicating that these memory cells are maintained in vivo for many years. The PBMC of young adults with no history of immunization against smallpox did not develop vaccinia-specific T cell responses after in vitro stimulation. Precursor frequency analysis of the vaccinia virus-specific memory CD4+ T cells from a donor immunized with vaccinia virus 35 years earlier revealed a frequency of 1 in 65,920 FACS sorted CD4+ T cells. We conclude that after immunization against smallpox in childhood, specific vaccinia virus T cell immunity can persist for greater than fifty years and may persist for the life of the individual in the presumed absence of exposure to vaccinia virus.

INTRODUCTION

The ability of a T cell to recognize a specific peptide epitope in the context of a major histocompatability complex (MHC) molecule on the surface of viral infected cells is provided by its T cell receptor (TCR) (1,2). The interaction between the TCR and the antigen-MHC complex triggers proliferation and clonal expansion of specific T cells (1,2). The proliferative T cell response continues during viral infections until the cells expressing the viral epitopes are eliminated or the virus becomes latent within cells and is no longer detectable by T cells. Some of the progeny of the antigen-responsive T cells develop into antigen-specific memory T cells. This subpopulation of T cells is maintained within the host and provides immune surveillance. In the event of reactivation of latent virus and expression of viral antigens, or a subsequent natural reexposure to the virus, specific memory T cells will become activated and clonally expand with greater magnitude than during the initial response. It is generally accepted that specific immune T cell memory persists after an encounter with antigen and may help to protect the immune host against subsequent exposure to that pathogen. The underlying mechanisms which contribute to immunological T cell memory are poorly understood and have only recently received much attention.

Vigorous and long-lasting protective immune responses have long been associated with smallpox vaccination. After a single immunization with vaccinia virus, virus specific CD4+ and CD8+ CTL are generated (3,4). Specific immunity is believed to be maintained for many years and booster immunizations are recommended every ten years for those at risk for infection. Due to the decreased risk of smallpox infection and side effects of vaccination, vaccinia virus has not been generally used since routine vaccinations were discontinued over 20 years ago. Laboratory workers using vaccinia virus and some members of the military continue to be vaccinated. Vaccinia virus does not cause

persistent or latent infections and therefore repeated endogenous antigenic stimulation is not thought to occur(5). These characteristics of vaccination against smallpox led us to investigate the longevity of the vaccinia virus-specific memory responses in humans.

Materials and Methods

Viruses: The New York City Board of Health strain of vaccinia virus (VAC) and three previously described recombinant vaccinia viruses (V/gag, V/pol, and V/gp160) expressing the gag, reverse transcriptase and envelope genes of HIV-1 (IIIB strain), respectively, kindly supplied by B. Moss, were used to infect target cells for CTL assays (4).

Preparation of target cells. B-lymphoblastoid cell lines (B-LCL) were established by Epstein-Barr virus transformation of each of the donors' PBMC. The B-LCL were either uninfected (control) or infected with vaccinia viruses at a multiplicity of infection of 10:1 for 12 to 16 h, labeled with sodium[^{51}Cr]chromate for 1.5 h, washed, and resuspended to give the appropriate concentration for use as target cells in cytotoxicity assays.

Preparation of effector cells. PBMC were obtained from the blood of asymptomatic HIV-1 antibody-positive individuals and HIV-1 seronegative donors by Ficoll-Hypaque density gradient centrifugation. To obtain T-cell lines, fresh PBMC from HIV-1 seropositive donors were seeded at 50 cells per well in 96-well plates with $2x10^5$ γ-irradiated allogeneic PBMC from normal donors in 0.2 ml of RPMI 1640 containing 10% fetal calf serum, recombinant human interleukin-2 (100 U/ml; Cellular Products, Buffalo, NY), and an anti-CD3 antibody 12F6 (0.1 ug/ml) kindly supplied by J. Wong, as previously described (7). Twice weekly, half of the medium was removed from each well and replaced with fresh medium. These cell lines were restimulated every 2 weeks and subsequently used as effector cells in the assay described below. Stimulation of PBMC from HIV-1 antibody

negative donors with live vaccinia virus was performed as previously reported (4).

Cytotoxicity assays. We used a ^{51}Cr-release assay as described previously (8). Briefly, assays were performed in triplicate wells, and the percent specific cell lysis was calculated by the formula 100 x (mean experimental release - mean spontaneous release)/mean total release - mean spontaneous release). The results of an assay were excluded if the mean level of spontaneous release was >30%.

Phenotypic Analysis and Cell Sorting. Cell lines were stained with fluorescein isothiocyanate-conjugated monoclonal antibodies (mAbs) anti-Leu4 (CD3), anti-Leu2 (CD8), anti-Leu3 (CD4), and anti-Leu11a (CD16) (Becton Dickinson, Mountain View, CA). The percentage of positively stained cells was analyzed by fluorescence-activated cell sorting (Becton Dickinson model 440). In preparation for experiments to determine precursor frequencies, CD4+ and CD8+ T cells were obtained by flow cytometry sorting. Briefly, PBMC were labeled for 30 min at 4°C with fluoresceinated anti-CD8+ antibody or phycoerythrin-coupled anti-CD4+ antibody (Becton Dickinson, Mountain View, CA). Large aggregates were removed by filtering through a 50 μm nylon mesh and the cells were immediately sorted. Sorting was also performed using PBMC gated on the basis of wide-angle and forward-angle light scatter values, which represented 60 to 70% of PBMC. The sorted CD8+ or CD4+ enriched populations were always greater than 98.0% pure.

Antibody and Complement Depletion Analysis. Anti-OKT3 (CD3), anti-OKT4 (CD4) and Anti-OKT8 (CD8) antibodies (Ortho Diagnostic Systems, Ind., Raritan, N.J.) and anti-Leu 11b (CD16; Becton Dickinson Co.) antibodies were used in complement depletion experiments. Vaccinia virus-stimulated effector cells were resuspended in 0.5 ml of RPMI 1640 supplemented with 2% FBS containing a 1:10 dilution of the antibody. Following 30 min of incubation at 4°C, the cells were washed with cold RPMI 1640 and suspended in 0.6ml to which 0.2ml of rabbit complement

(Cedarlane Laboratories, Hornby, Ontario, Canada) was added. After 1h of incubation at 37°C, the cells were washed three times and used as effector cells in cytotoxicity assays.

Limiting Dilution Assays for Precursor Frequency Determination. Microcultures were initiated under limiting dilution conditions with 5,000 to 60,000 sorted CD4+ T cells per well in 24 replicate wells. Each microculture received $2x10^5$ γ-irradiated autologous PBMC in 200 µl RPMI 1640 medium supplemented with 20% Fetal Calf Serum, 40U r-IL2/ml, and vaccinia virus antigen. Vaccinia antigen was prepared by infecting a confluent monolayer of CV-1 cells with concentrated vaccinia virus which was then harvested by cell scraping 24 h after incubation. After a freeze-thaw, sonication was performed and the cell-virus extract was boiled for 10 min to inactivate any residual infectious virus before use. At three day intervals one-half of the culture media was removed and replaced with fresh media without vaccinia antigen as described above. On day 10, each well was split and cells were assayed for cytotoxicity on autologous uninfected B-LCL, or B-LCL infected with vaccinia virus. Individual wells were considered positive if the calculated specific lysis of the virus infected target cells was greater than 3 standard deviations of the mean levels of lysis calculated from negative wells. Precursor frequencies were calculated using χ^2 analysis according to Taswell (9), using a computer program kindly provided by Dr. Richard Miller (U. of Michigan, Ann Arbor, MI).

Results

PBMC of HIV-1 seropositive donors exhibited vaccinia virus-specific cytotoxic activity against autologous B-LCL expressing vaccinia antigen. Cryopreserved PBMC from 22 asymptomatic HIV-1 seropositive donors and 8 HIV-1 seronegative healthy donors were tested for HIV-1 envelope antigen-specific cytotoxicity, using autologous B-LCL target cells that were either uninfected,

infected with VAC, or the recombinant V/gp160 as described in Materials and Methods. Among the HIV-1 seropositive donors, we detected significant HIV-1 envelope antigen-specific cytotoxicity in 2(9%) of the subjects cultured PBMC that were tested (data not shown). Significant lysis of target cells expressing vaccinia antigens was observed using the cultured PBMC of 6 HIV-1 seropositive donors as shown in Table 1. The K562 cell line was used as a target cell to measure natural killer cell activity.

Establishment of vaccinia virus-specific CD4+ CTL lines. Several cell lines were obtained that specifically lysed vaccinia virus-infected target cells after stimulation of isolated PBMC with γ-irradiated allogeneic PBMC, anti-CD3 monoclonal antibody, and recombinant human IL-2. These cell lines were expanded and subcloned by limiting dilution at 0.3, 1, or 3 cells per well. We isolated vaccinia virus-specific cytotoxic T cell clones from each of three HIV-1 seropositive donors (Table 2). These cell lines lysed autologous target cells infected with vaccinia virus, and the lysis of K562 cells was low. Phenotypic analysis of these CTL lines revealed that they were all CD3+ CD4+ CD8- Leu11-.

Demonstration of long-lived vaccinia virus specific CD8+ CTL responses. Presumably, the HIV seropositive donors from whom the CD4+ clones were generated had not been exposed to vaccinia virus since primary childhood vaccination, because the donors were over 30 years of age, had not served in the military and had not been vaccinated since early in childhood. To determine whether vaccinia virus-specific CTL precursors are maintained the peripheral blood of individuals for long periods of time after a single primary immunization in early childhood, two healthy HIV-1 antibody negative donors, VA15 and VA16, were identified who were known to have been immunized as children with vaccinia virus 35 and 50 years earlier, respectively. These donors stated that they have

had no subsequent exposure to vaccinia or to any other poxvirus. We stimulated these two donors' PBMC with live vaccinia virus in vitro in an attempt to detect vaccinia virus specific memory CD8+ CTL because we had only isolated CD4+ MHC class II restricted CTL from the HIV-1 seropositive donors using anti-CD3 and rIL-2 stimulations. This method of stimulation with live vaccinia virus had been established by us and used successfully to generate vaccinia virus specific CD8+ MHC class I restricted CTL in vitro (4).

Donor PBMC were stimulated in vitro with live vaccinia virus which induced a low level of vaccinia virus specific proliferation and the cultures were restimulated with anti-CD3 on day 7. On day 14 the cultures were restimulated with live vaccinia virus and were assayed on day 21 for cytotoxic activity. The bulk cultured cells derived from donors VA15 and VA16 lysed vaccinia virus-infected autologous B-LCL at 50.8% and 44.2% respectively, at an E/T ratio of 50:1 (Table 3). Incubation of effector cells with anti-CD3+ or anti-CD8+ specific monoclonal antibodies in the presence of complement reduced the levels of lysis significantly, indicating that CD8+ CTL were the major effector cells in this virus-stimulated population. There was some decrease in the level of lysis after treatment with anti-CD4+ and complement, suggesting a contribution by vaccinia virus specific CD4+ CTL(Table 3).

To confirm that the vaccinia virus specific CTL activity observed in these cultures was not a result of primary in vitro stimulation, the PBMC of two healthy young adult donors who had no history of immunization with vaccinia virus were used as controls. The PBMC from these donors, VA21 and VA23, were stimulated in an identical fashion to the PBMC of donors VA15 and VA16 and were assayed on day 21 for vaccinia virus-specific CTL activity and had none (Table 3).

Frequency analysis of vaccinia virus-specific CTL memory cells. As described above, the PBMC

isolated from donor VA15 who had been immunized with vaccinia virus more than 35 years ago exhibited vaccinia virus-specific CTL activity after stimulation in vitro. We wanted to determine the frequency of vaccinia virus-specific memory T cells within the PBMC of this donor. We assumed that the precursor frequency of the vaccinia virus specific CTL would be low, so preliminary experiments were done to ensure that we could detect a positive response. Sorting of donor PBMC by FACS gave rise to a relatively pure population of CD4+ T cells and enabled us to assay for CD4+ vaccinia virus-specific memory T cells. LDA analysis was performed using freshly sorted PBMC. Analysis of the precursor frequency of CD4+ vaccinia virus-specific memory T cells resulted in a calculated frequency of 1 in 65,920 sorted CD4+ T cells (Figure 1). Three experiments were performed on the sorted CD4+ T cells and the calculated frequencies for each experiment were consistent, differing by less than 2%.

DISCUSSION

In this communication, we report on the duration of vaccinia-virus specific CTL memory responses in humans. Vaccinia virus-specific CD4+ CTL clones were isolated from the PBMC of HIV-1 seropositive individuals many years after vaccinia immunization in early childhood. We then studied vaccinia virus-specific CTL memory responses of healthy donors and found that both CD4+ and CD8+ memory T cell responses had persisted for greater than fifty years (Donor VA16) after exposure to vaccinia vaccine in early childhood. We determined that the precurser frequency of vaccinia virus-specific CD4+ CTL from a donor 35 years after vaccination was 1 in 65,920 sorted CD4+ T cells or approximately 3 in $1x10^6$ PBMC. This calculated frequency is lower than the reported precurser frequencies for Varicella-zoster, HIV-1, and cytomegalovirus specific T cells (10,11,12), but those viruses cause persistent and/or latent infections unlike vaccinia virus.

The long-lived T cell memory responses we observed are striking; however, a recent study also suggested that memory T cell responses to vaccinia virus may be long lasting. In a randomized phase 1 trial reported by Cooney, et al., 35 healthy HIV-1 seronegative young adult males, 31 of whom had a history of smallpox immunization and 4 of whom were vaccinia naive, were immunized with a recombinant vaccinia vaccine expressing the gp160 envelope gene of HIV-1 (13). Individuals who had been immunized as young children with vaccinia had poor immune responses to the HIV-1 gp160 antigen compared to those who had no previous exposure to vaccinia. The results from this trial suggested that long lasting immunity to vaccinia limits the replication of the vaccinia recombinant virus used for immunization (13). The observations made during that study, together with data reported recently on the use of vaccinia recombinants expressing HSV gene products in mice (14,15), are consistent with our results showing vaccinia virus-specific memory T cell responses are long

lasting and may persist for life.

The results from the complement depletion assay using bulk cultured cells demonstrated that CD8+ memory CTL activity was dominant in short-term virus stimulated bulk cultures. We expected the precursor frequency of the CD8+ memory T cell population to be greater than that observed for the CD4+ T cells; however, we found it difficult to determine the precursor frequency of CD8+ memory T cells. The number of CD8+ cells isolated by FACS was only 1/3 the number of CD4+ T cells obtained, so fewer CD8+ T cells were available for these assays and our culture conditions with live virus may not have been optimal for detection of relatively low numbers of CD8+ T cells. Others have reported failures to detect vaccinia virus-specific T cell responses in human donor PBMC in vitro (16,17), but we found that live virus was needed as the in vitro stimulant to detect vaccinia virus CD8+ CTL in bulk culture (4).

The underlying mechanisms which contribute to immunological memory are poorly understood and have only recently received much attention. The major question with respect to persistent immunological T cell memory is : How is it maintained in vivo ? There is controversy about whether the maintenance of memory T cells requires periodic interaction with antigen presenting cells expressing the relevant peptide (18,19), or that T cell "memory " might be maintained in the absence of specific antigen stimulation (20,21). Oehen, et al., reported that the adoptive transfer of immune spleen cells into syngeneic recipient mice required the presence of viral antigen for the maintenance of the antiviral protective capacity of the transferred cells (18). Matzinger and Gray reported similar results (19). Other reports challenge those findings (20,21). Lau, et al., used adoptive transfer experiments in the LCMV mouse model and reported that memory CD8+ CTL persist and retain the memory phenotype indefinitely in the apparent absence

of priming antigen, and these CTL apparently protected mice for up to 2 years against virus challenge (20). Mulbacher utilized a similar approach in a mouse model of influenza virus and also concluded that CTL memory is long-lived in the apparent absence of antigen (21). Our results using PBMC of adult humans following immunization with vaccinia virus in early childhood also suggest that persistence of antigen is not required for the long-termed maintenance of T cell memory. Although there is no evidence for the persistence of vaccinia virus or antigens in vivo, dendritic cells may sequester antigen for periods of time making it available for persistent stimulation of the immune system (22); however, further studies are needed to clarify the potential contribution dendritic cells may have in the maintenance of T cell memory.

If antigen persistence is not required, what other mechanisms contribute to long-lived specific T cell memory? One mechanism that has been suggested is immunological cross-reactivity between viruses (23,24,25). Selin, et al., used percursor frequency analyses to study virus cross-reactive T cell responses in mouse models and postulate that exposure to one virus might provide a boost in immunity to an unrelated virus (23). They could not rule out the possibility of enhanced non-specific stimulation by lymphokines generated during the immune response to the heterologous virus, but their results tend to support the cross-reactivity hypothesis. In the absence of significant homology among unrelated viruses, they suggested that the observed T cell cross reactivity may be due to crossreactive epitopes possessing major amino acid differences but having discrete critical residues in common (23). This hypothesis may be reasonable in light of what is known about the phenotype of memory T cells (26,27). When T cells aquire a "memory" phenotype, they upregulate the expression of several surface adhesion molecules in addition to the IL-2 receptor, and become more sensitive to stimulation by a low affinity T cell specific peptide (23,24). This "promiscuous" behavior may allow a memory

T cell to become activated through an interaction of the T cell receptor with an APC presenting a peptide epitope from a different virus than the virus that induced the memory response. Shimojo, et al., characterized a T cell line generated against an influenza virus encoded peptide which specifically recognized a dissimilar rotavirus derived peptide (28), which supports this hypothesis. There are an increasing number of examples of immunological cross reactivity between proteins of infectious organisims and human proteins (29), and this molecular mimicry at the peptide level may play a role in T cell crossreactivity in vivo. The interaction of T cells with APC presenting self peptides which mimic epitopes on infectious agents may also stimulate their propagation. Thus, memory T cells may be promiscuous in their ability to recognize varying peptides and may be stimulated in a cross-reactive fashion.

The data we present are perhaps the first clear evidence that virus-specific T cell memory can persist for greater than fifty years in humans in the presumed absence of antigen. We believe that human subjects with prior defined exposure to vaccinia virus years earlier provide an excellent model for the study of human T cell memory. Further elucidation of the underlying mechanisms which contribute to the maintenance of T cell memory will have an impact on our understanding of the basis of immunological memory and on the design of future vaccines.

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Table 1. Vaccinia virus-specific cytotoxic activity in cultured PBMC of asymptomatic HIV-1 seropositive donors[a]

% Specific Lysis of target cells				
Donor No.	Uninfected	VAC	V/gp160	K562
2	17.2	29.8	23.8	1.8
11	8.9	31.8	29.0	1.7
12	10.1	25.9	18.4	2.3
13	2.8	13.5	15.4	2.1
26	7.0	17.4	13.1	2.5
27	2.3	17.8	15.4	3.3

[a]Cytotoxicity was determined as described in Materials and Methods in a 6 hr^{51} Cr release assay, using four different E/T ratios. Values shown here are at a representative E/T ratio of 50:1.

Table 2. Lysis of vaccinia virus-infected target cells by T cell clones from HIV-1 seropositive donors

Donor No.	Clone No.	Uninf.	% Specific lysis of target cells				
			VAC	V/gag	V/pol	V/gp160	K562
11	214	0	71.8	62.8	64.8	73.5	10.4
2	140	3.1	27.7	29.6	29.3	12.6	9.8
12	109	11.4	32.8	26.5	NT	18.5	3.3

$2x10^3$ target cells were incubated with effector cells at an E:T ratio of 4:1 for 5 h. Percent specific ^{51}Cr release was calculated by the formula described in Materials and Methods.

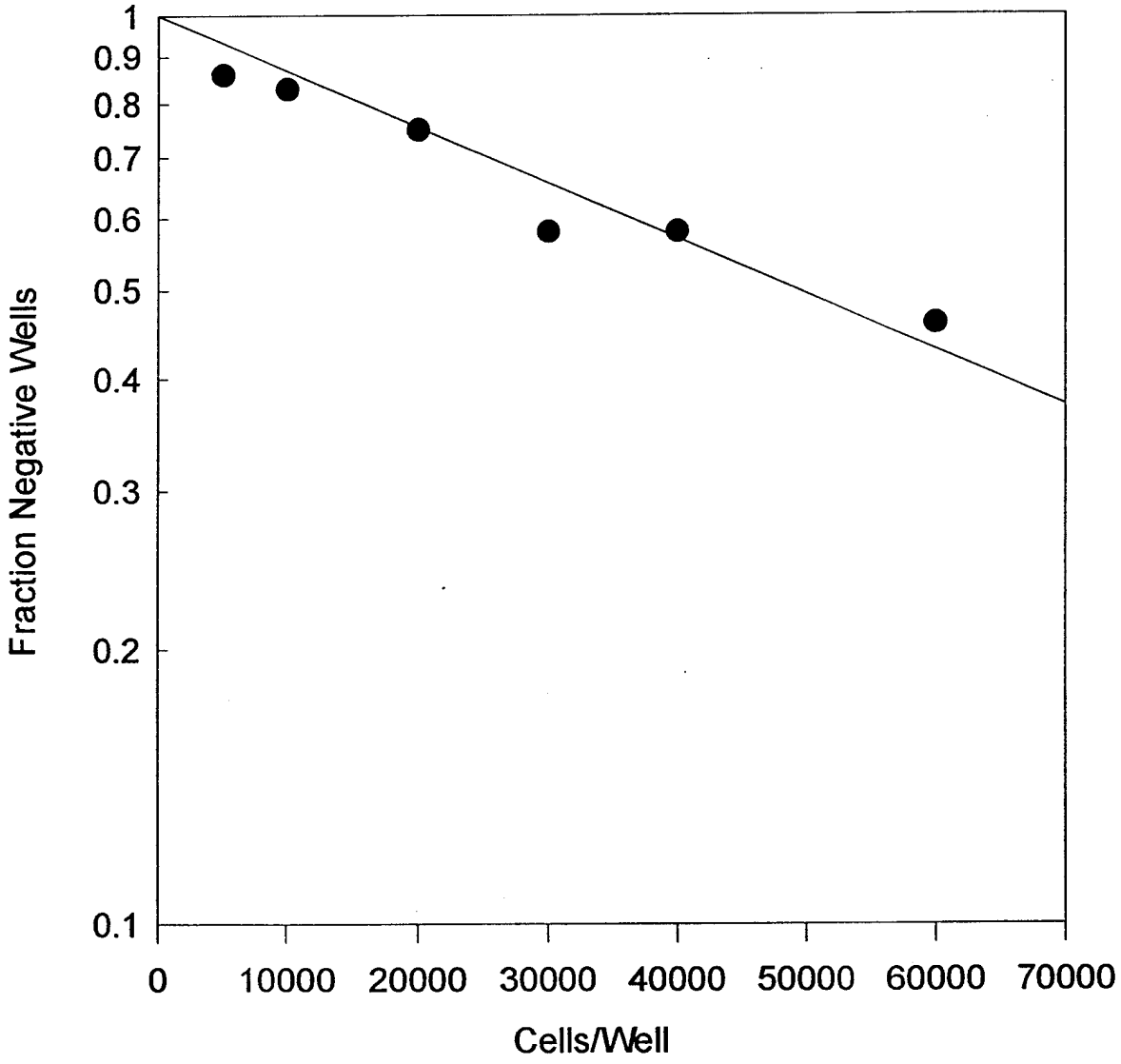
Table 3. Characterization of bulk culture cytotoxicity activity

Donor(day), treatment	% specific ^{51}Cr release from target cells[c]		
	B-LCL	VAC[a]	K562
VA15 (21),			
Complement	-0.2	50.8	23.1
Anti-CD4+ complement	-0.3	32.9	14.7
Anti-CD8+ complement	-3.3	-5.5[b]	9.8
Anti-CD3+ complement	-3.4	-5.1	2.7
Anti-CD16+ complement	3.8	37.4	13.0
VA 16 (21),			
Complement	3.3	44.2	35.3
Anti-CD4+ complement	0.0	36.9	27.3
Anti-CD8+ complement	1.7	3.4	19.0
Anti-CD3+ complement	-1.3	-0.2	15.7
Anti-CD16+ complement	2.3	36.2	25.9
VA21 (21)			
Complement	-1.9	-3.5	0.3
Anti-CD3+ complement	-1.1	-4.9	1.1
Anti-CD16+ complement	-2.8	-2.7	0.4
VA23 (21)			
Complement	3.2	7.2	-0.1
Anti-CD3+ complement	0.6	3.3	-0.7
Anti-CD16+ complement	3.6	2.3	-0.2

a Autologous B-LCL infected with vaccinia virus
b Underlined values are those which were significantly decreased by treatment
c E/T Ratio 50:1

Figure Legends

Figure 1. Frequency of vaccinia virus specific lytic effectors among sorted CD4+ lymphocytes. The sorted CD4+ T cells were stimulated with vaccinia virus antigen as described in Materials and Methods. Lytic activities were measured in a CTL assay on day 10. The frequency of vaccinia virus specific CD4+ CTL in donor VA15 was calculated as 1/65,920.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30000
40000
50000
60000
70000
Cells/Well